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# Suitability and limitations of methods for characterisation of activity of malto-oligosaccharide-forming amylases

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**Abstract**

The suitability and limitations of essential methods and reference substrates used for characterisation of activity of amylolytic enzymes is investigated. Saccharogenic, chromogenic and chromatographic methods are included. The results are discussed in relation to the measurement of reaction rates, determination of action mode and product specificity and the impact on identification and nomenclature of malto-oligosaccharide-forming amylases. An accurate determination of reaction rates using the saccharogenic methods strongly depends on the degree of polymerisation (DP) of the standards used and the hydrolysis products formed by the amylase. Particularly the use of glucose as standard can lead to overestimates due to the differences in the reducing potential of glucose and malto-oligosaccharides. The reliability of the chromogenic methods for determination of action mode depends on the DP of the substrate and the specificity of the amylase. For a characterisation of the starch hydrolysis products and the variation in the DP during hydrolysis, high performance anion-exchange chromatography with pulsed amperometric detection provided a fast and reliable method. A literature survey revealed varying and inconsistent use of nomenclature of malto-oligosaccharide forming amylases. Therefore a systematic approach identifying three main classes of activity is suggested using not only the mode of action and the DP of the major product but also the stage of hydrolysis at which this product is formed. 

*Keywords:* Malto-oligosaccharide-forming amylase; Starch hydrolysis products; Saccharogenic and chromogenic methods; High-performance anion-exchange chromatography with pulsed amperometric detection

## 1. Introduction

Starch is one of the most abundant polysaccharides in nature where it serves as energy storage in plant seeds and tubers [1]. Starch is a homopolymer of glucose consisting of an essentially linear fraction termed amylose with glucose linked by α-D-(1→4) O-glucosidic linkages and a highly branched fraction termed amylopectin with linear segments of α-D-(1→4) O-glucosidic linked residues and branch points at α-D-(1→6) O-glucosidic linkages. The linear amylose molecule contains one reducing end (one free anomeric carbon atom) and one non-reducing end, while an amylopectin molecule has one reducing end and numerous non-reducing ends due to the branched structure. In order to access the chemical energy stored in starch, organisms need starch-hydrolysing enzymes.

Enzymes capable of hydrolysing starch are denoted amylolytic enzymes and are classified as hydrolases which cleave α-D-(1→4) O-

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glucosidic or α-D-(1→6) O-glucosidic linkages. Amylolytic enzymes have been divided into several subclasses according to their activities on starch [2,3]. The distinction has been made according to the configuration of the anomeric carbon atom in the products, i.e. α- or β-amylases or action mode on the substrate, i.e., endo- or exo-activity (Table 1). The α-amylases (E.C. 3.2.1.1) are endo-acting enzymes that randomly attack the internal α-D-(1→4) O-glucosidic linkages of starch except for those adjacent to the ends of the substrate and those in the vicinity of branch points. The end products are α-limit dextrins, which are branched saccharides not prone to further hydrolysis and malto-oligosaccharides of varying degrees of polymerisation (DP), characteristic of the particular enzyme [2,3]. β-Amylases (E.C. 3.2.1.2) are exo-acting amylases which hydrolyse α-D-(1→4) O-glucosidic linkages in starch from the non-reducing end of the substrate, leading to β-maltose and β-limit dextrins. α-Glucosidases (E.C. 3.2.1.20) and glucoamylases (E.C. 3.2.1.3) are also exo-acting amylases producing α-D-glucose and β-D-glucose, respectively [2–4]. Glucoamylases also hydrolyse α-D-(1→6) O-glucosidic linkages, however at a slower rate than the α-D-(1→4) linkages. Debranching enzymes, which can hydrolyse the α-D-(1→6) O-glucosidic linkages in amylopectin are pullulanases (E.C. 3.2.1.41) and isoamylases (E.C. 3.2.1.68). Closely related to α-amylases are cyclodextrin glycosyltransferases (CGTases, E.C. 2.1.4.19). The action on starch of CGTases results in the formation of cyclic malto-oligosaccharides of six or more glucose units [4,9], while a lower rate of hydrolysis activity is found concomitantly.

Several bacterial amylases capable of degrading starch to malto-oligosaccharides of a certain DP have been identified. Traditionally they are termed product-specific amylases and include amylases forming either maltotriose, maltotetraose, maltopentaose, or maltohexaose as the major product [2–4,10]. The pure malto-oligosaccharides have mainly been used as fine chemicals and for diagnostic kits [10,11] and due to the difficulties in preparative separation of malto-oligosaccharides a considerable interest in amylases forming malto-oligosaccharides of a specific DP has evolved. As the product size distribution is developing during the time of hydrolysis some confusion related to the properties and the nomenclature of these amylases has resulted.

The present investigation is a study of the suitability and limitations of essential methods and reference substrates used to characterise amylolytic activity regarding substrate conversion, action mode and product specificity. The results are discussed in relation to the identification and nomenclature of malto-oligosaccharide-forming amylases.

Table 1
Starch hydrolysing enzymes and their mode of action on starch.

| Enzyme name | Mode of action | Glucosidic linkages attacked | Main product(s) formed | Ref. |
|---|---|---|---|---|
| α-Amylase E.C. 3.2.1.1) | Endo | α-D-(1→4) | mixture of malto-oligosaccharides with α-configuration | [2–4] |
| β-Amylase (E.C. 3.2.1.2) | Exo | α-D-(1→4) | β-maltose | [2–4] |
| α-Glucosidase (E.C. 3.2.1.20) | Exo | α-D-(1→4) | α-glucose | [2–4] |
| Glucoamylase (E.C. 3.2.1.3) | Exo | α-D-(1→4) and α-D-(1→6) | β-glucose | [2–4] |
| Isoamylase (E.C. 3.2.1.68) | Endo | α-D-(1→6) | linear malto-oligosaccharides up to 25 glucose units without branches | [2–4] |
| Pullulanase (E.C. 3.2.1.41) | Endo | α-D-(1→6) | various linear malto-oligosaccharides | [2–4] |
| Exo-maltotetrao-hydrolase (E.C. 3.2.1.60) | Exo | α-D-(1→4) | maltotetraose | [5,6] |
| Exo-maltohexao-hydrolase (E.C. 3.2.1.98) | Exo | α-D-(1→4) | maltohexaose | [6–8] |

Table 2
Methods for determination of amylolytic activity, chemical reaction and detection method

| Method | Substrate | Reaction principle | Detection | Ref. |
|---|---|---|---|---|
| *Saccharogenic methods* | | | | |
| Ferricyanide | native or soluble starch | alkaline ferricyanide(III) reduced to ferrocyanide(II) | spectrophotometric | [14,15] |
| Somogyi -Nelson | native or soluble starch | alkaline cupri(II) ions reduced to cupro(I) ions, colour with arsenomolybdat | spectrophotometric | [16,17] |
| DNS | native or soluble starch | alkaline 3,5-dinitrosalicylic acid reduced to 3-amino-5-nitrosalicylic acid | spectrophotometric | [18] |
| $CuSO_4$– biquinoline | native or soluble starch | alkaline cupri(II) ions reduced to cupro(I) ions, colour with 4,4′-dicarboxy-2,2′-biquinoline | spectrophotometric | [19] |
| *Chromogenic methods* | | | | |
| Cerealpha™ | BPNPG7 [a] | endo-attack followed by glucoamylase/α-glucosidase action on *p*-NP part release *p*-NP | spectrophotometric | [20,21] |
| Betamyl™ | PNPG5 [b] | exo-attack and α-glucosidase action on *p*-NP part releases *p*-NP | spectrophotometric | [22] |
| Dyed-starch | AZCL –amylose [c] | release of blue colour when amylose is hydrolysed | visually or spectrophotometric | [12] |
| *Amyloclastic methods* | | | | |
| Iodometric | starch or amylose | amylose–$I_3^-$ complex (blue) hydrolysis decreases the complex and the blue colour | spectrophotometric | [23,24] |
| Viscosity | starch in high concentrations | hydrolysis results in a decrease of the solution viscosity | viscosity measurement | [4] |

[a] Blocked *p*-nitrophenolmaltoheptaose.
[b] *p*-nitrophenolmaltopentaose.
[c] Azo-colour crosslinked amylose.

## 2. Results and discussion

*Determination of enzyme activity.*—The most commonly used methods for activity determination of amylases can be grouped into saccharogenic, chromogenic and amyloclastic methods [12]. Amyloclastic methods measure the degradation of starch by viscometric, turbidimetric, iodometric or nephelometric procedures [13]. The saccharogenic methods are a direct measure of hydrolysis which determine the number of reducing ends formed [14–19] and the chromogenic methods, which include the use of coloured substrates [12,20,21]. The mechanism of the different assays is summarised in Table 2. These methods do not qualitatively determine the actual products formed and therefore care should be taken when the activity of malto-oligosaccharide-forming amylases is to be measured.

*Saccharogenic methods.*—It is evident, that during hydrolysis of α-(1→4) glucans the number of reducing ends increases, except for the activity of CGTases, which mainly result in cyclic products. Kinetic studies of amylase-catalysed reactions is therefore obtained by saccharogenic methods where the number (or molar concentration) of glucosidic linkages hydrolysed in a certain time is determined [4,13]. An increase in the amount of reducing ends released is determined by oxidation of the anomeric carbon atom at the reducing end of the hydrolysis product. A corresponding reduction of a metal ion or a compound imparts colour either in the reduced state itself or as a complex with a ligand (Table 2). The colourimetric measurement of the formation of reducing ends has been accomplished at alkaline conditions primarily by the use of three different oxidising reagents: copper

[16,17,19], ferricyanide [14,15] and 3,5-dinitrosalicylic acid [18,25].

For quantification of the number of reducing ends formed the most common reference used is maltose, particularly if malto-oligosaccharides or dextrins are the products formed [14]. The standard curves obtained with glucose, maltose and maltotriose by the use of the $CuSO_4$/biquinoline assay [26] and the DNS method [18] are illustrated in Fig. 1. The $CuSO_4$/biquinoline assay was found to be sensitive to small concentrations of products formed in the range 0.025–0.25 mM (Fig. 1(A)). The DNS method could be used at concentrations of 0.5–3.0 mM (Fig. 1(B)) and the curves were still found to be linear up to 10 mM. Identical concentrations of glucose and maltose did not contribute equally to the reducing power detected by the two assays. An overestimation of the amylase activity would occur when glucose instead of maltose was used as reference. Particularly for the DNS method the use of maltotriose as a reference resulted in a curve different from the maltose standard. For the $CuSO_4$/biquinoline assay a curve similar to the one for maltose was obtained with maltotriose, maltotetraose and maltoheptaose. Investigations of the Somogyi–Nelson method [16,17] showed that glucose and maltose as references did not give rise to different curves (data not shown). These results were confirmed by the findings that the copper procedure [16] gives equimolar reducing equivalents for equimolar reducing ends of malto-oligosaccharides.

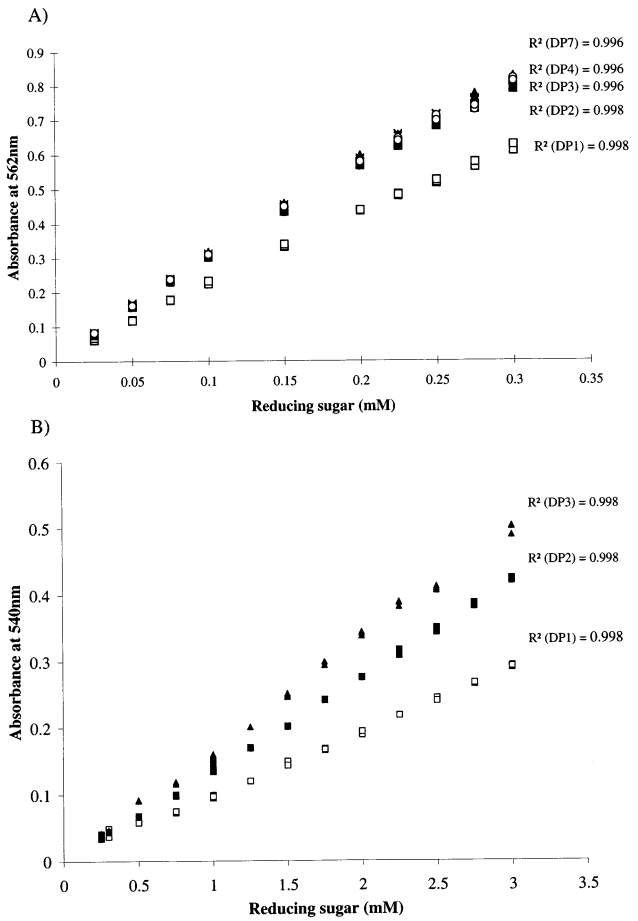


Fig. 1. Standard curves for determination of reducing sugar equivalents by saccharogenic assays using glucose and malto-oligosaccharides. Assay: (A) $CuSO_4$/biquinoline assay, (B) DNS assay, the linear correlation coefficient ($R^2$) are seen. □ glucose, ■ maltose, ▲ maltotriose, X maltotetraose, and ○ maltoheptaose.

Studies of the ferricyanide method also revealed a higher reducing potential of glucose than malto-oligosaccharides [14]. The method was modified by Hizukuri et al. [15] to obtain the same reducing effect of glucose and malto-oligosaccharides up to DP 40. The dinitrosalicylic acid reagent gives a decreasing reducing potential with an increase in the number of D-glucose units in the oligosaccharide chain [4,27,28].

Malto-oligosaccharide-forming amylases have been found to form either maltotriose, maltotetraose, maltopentaose or maltohexaose as the dominating product. As D-glucose and malto-oligosaccharides do not have the same effect on the oxidising reagents of most saccharogenic assays, we found that D-glucose is inappropriate for use as a reference. A correct determination of the activity would be possible only by the use of the predominant hydrolysis product as reference for the saccharogenic assay. Even a mixture of products can result in a misleading activity determination.

*Chromogenic methods.*—For the chromogenic methods, substrates such as insoluble substrate-dye complexes, which liberate soluble dye fragments upon hydrolysis [12] or *p*-nitrophenol (*p*-NP) derivatives of malto-oligosaccharides, which release the *p*-NP upon hydrolysis [22], are used. *p*-NP derivatised malto-oligosaccharides (maltopentaose, maltohexaose or maltoheptaose) are hydrolysed by the amylases in conjunction with α-glucosidase or glucoamylase leading to the liberation

of *p*-NP and measured spectrophotometrically as the yellow coloured nitrophenoxide ion at weakly alkaline conditions [20–22]. Due to the need of α-glucosidase for the assay of *p*-NP-derivatised malto-oligosaccharides the procedure is limited to a pH range of 5.0–6.0. For the endo-acting amylases the determination of activity can be achieved by the use of a *p*-NP-maltoheptaoside blocked with a 4,6-*O*-benzylidene at the non-reducing end according, e.g., to the Cerealpha method [21] (see Table 2). The blocking of the non-reducing end prevents activity of exo-amylases. For the determination of exo-activity a non-blocked mixture of *p*-NP-maltoheptaoside, *p*-NP-maltohexaoside and α-glucosidase is used as, e.g., in the Betamyl method [22] (see Table 2). Strict exo-activity on a starch substrate leading to the formation of maltoheptaose (DP7) as the end product would result in limited or no cleavage at all of the *p*-NP-maltopentaose substrate depending on the subsite affinities.

Enzyme activity on short substrates (e.g. maltopentaose) may not lead to results useable to predict the activity on polymeric substrates as native starch which could vary in structure and be branched. The substrate can indeed be too short for detection of the actual activity, which is important to consider when using *p*-NP-derivatised malto-oligosaccharide substrates. Furthermore, the activity of the enzyme can vary depending on the length of the substrate as known from α-glucosidases, which show a lower activity on long substrates than on short substrates [2].

Amylolytic activity can be determined using insoluble substrates with covalently bound dyes. The insoluble part can be either starch, amylose or amylopectin [22] e.g. the azo-colour crosslinked amylose termed AZCL–amylose (Table 2). The dye is water soluble and is liberated as a result of hydrolysis. However, the size of the starch hydrolysis product or the cleavage mechanism cannot be determined by this method.

*Amyloclastic methods.*—The amyloclastic methods include the iodometric method (the so-called starch–iodine blue colour method) and the measurement of the changes of the viscosity of a starch solution. The iodometric method is performed by addition of an $I_2$–KI solution to time course samples withdrawn from starch or amylose solutions added to the actual amylase [23]. The amylose helix forms an inclusion complex with iodine and triiodide ($I_2$ and $I_3^-$) which results in a blue colour [24,29]. When the starch or amylose is cleaved by amylases the substrate decreases in size, which also decreases the size of the complex and the intensity of blue colour. John et al. [24] found an acrolimit at which no colour was detected for linear chains of 20 glucose units or less. Moreover, helices in amylopectin do not contribute to the blue colour obtained for 39 glucose units or more [24]. The stability of the formed iodine–starch complex depends on the temperature and pH [30]. In the present investigation analysis of the intensity of starch–iodine blue colour during hydrolysis was performed at room temperature and constant pH.

The starch–iodine blue colour method can be used for the determination of the relative activity of an amylase [13]. To investigate the hydrolysis action of three different amylases, the progress in starch hydrolysis was followed by simultaneous starch–iodine blue colour formation and liberation of reducing ends (see Fig. 2). A steep slope is the result of a fast reduction of the starch–iodine blue colour due to random endo-activity by the α-amylase (BAN, slope, $a = -26$). The maltogenase and the extracellular enzyme preparation from *Pseudomonas stutzeri* NRRL B 3389 showed evidence of a prevalent exo-activity indicated by a flat slope of $a = -2$ and $a = -12$, respectively (see Fig. 2).

Direct comparison of reaction rates of a maltose and a malto-oligosaccharide-forming ($DP > 2$) exo-amylase by the use of a saccharogenic assay may be difficult since the expected result would be fewer reducing ends released by the latter during the progress of the reaction. The hydrolysis activity of CGTases cannot be detected by any of these assays since they only produce low amounts of reducing ends. Furthermore, the cyclodextrins formed by CGTases complex with iodine and interfere with the starch–iodine method [26]. The information obtained from the saccharogenic assays, the chromogenic assays, or the

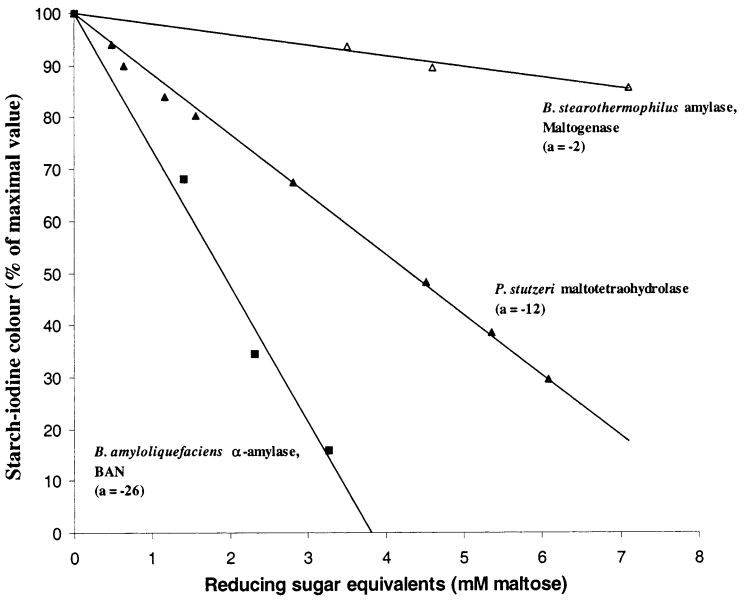


Fig. 2. Determination of endo- and exo-action mode of some enzyme preparations. The formation of starch–iodine blue colour is plotted against the production of reducing sugar equivalents. The slope of the linear curve (a) indicates the prevalence of endo- or exo-action mode.

Table 3
Selected properties of the TLC, HPLC and HPAEC–PAD methods for detection of starch hydrolysis products formed by amylase activity [a]

| Method | Pretreatment | Reproducibility | Analysis time | Separation of products | Cleaning procedure | Use of a gradient | DP-separated |
|---|---|---|---|---|---|---|---|
| TLC | ++ | ++ | +++ | ++ | +++ | − | 10 |
| HPLC | ++ | +++ | +++ | ++ | ++ | + | 20 |
| HPAE–PAD | +++ | +++ | +++ | +++ | +++ | +++ | 50 |

[a] −, impossible; +, hardly possible or not easy; ++, can be done, fair; +++, easy, good.

starch–iodine blue colour method, cannot reveal which starch hydrolysis product is predominantly formed.

*Analysis of the starch hydrolysis products.*— For determination of the starch hydrolysis products formed by the malto-oligosaccharide-forming amylases, chromatographic methods using different separation techniques have been applied. These methods show different advantages and limitations for identification of the starch hydrolysis products.

Thin-layer chromatography (TLC) is a low cost method and still commonly used as an analytical tool for the detection of starch hydrolysis products because of its simplicity and relatively high sensitivity [5,7,31–36]. However, the variation on a single analysis as well as between analyses can occur as a result of variation in application of samples to the TLC-plate and also temperature, and the conditions for colour detection and development including distribution of the reagent (see Table 3).

High pressure liquid chromatography (HPLC) with refractive index (RI) detection does not easily provide a stable baseline and is sensitive to the choice of eluent and sample matrix components [37]. Silica-based columns must be operated at a pH < 7.5 and therefore, saccharides with high DP, which are soluble only in alkaline solution cannot be analysed on these types of column. Separation of malto-oligosaccharides up to DP 20 by adsorption chromatography on chemically bonded phases [38] has been achieved (see Table 3).

The development of high performance anion-exchange chromatography (HPAE) com-

bined with pulsed amperometric detection (PAD) has provided a highly improved separation of malto-oligosaccharides [39,40] and increased the sensitivity of detection significantly. For a homologous malto-oligosaccharide series, it can be generalised that the larger DP, the longer the retention time (see Fig. 3(C)). However, the response for similar molar concentrations of different malto-oligosaccharides decreases with increasing molecular size until DP6 (Fig. 4).

Different amylases give rise to different starch hydrolysis products and malto-oligosaccharide-forming amylases give rise to major amounts of one single product. The initial starch hydrolysis products obtained with amyloglucosidase from *Aspergillus niger* (E.C. 3.2.1.3), maltogenase from *Bacillus stearothermophilus* (E.C. 3.2.1.-), α-amylase from *Bacillus amyloliquefaciens* (BAN, E.C. 3.2.1.1), CGTase from *Paenibacillus* sp. F8 (E.C. 2.4.1.19), and exo-maltotetraohydrolase from *P. stutzeri* NRRL B 3389 (E.C. 3.2.1.60) are shown in Fig. 3. It is clearly indicated that malto-oligosaccharide-forming amylases can be detected by the sole hydrolysis product formed (Fig. 3(B, E)). Even CGTases give rise to a characteristic chromatogram indicated by the irregular peak shapes of β-CD and γ-CD in Fig. 3(D). Furthermore, cyclodextrins display approximately 11 times lower response on a molar basis, than malto-oligosaccharides [41].

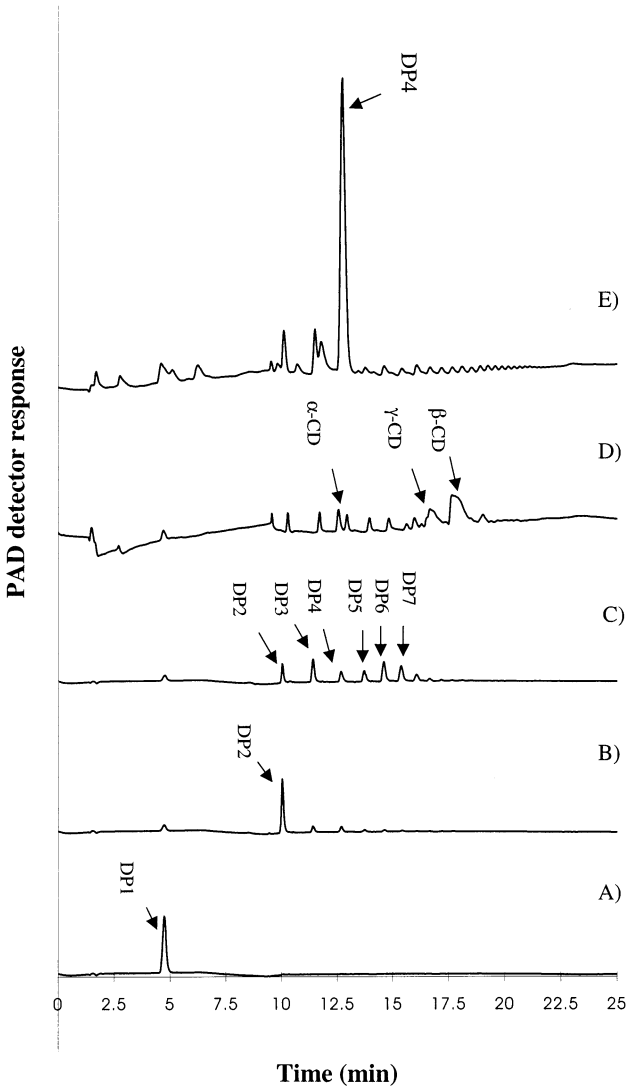


Fig. 3. HPAE–PAD chromatograms obtained after starch hydrolysis with different amylolytic enzymes. (A) amyloglucosidase, (B) maltogenase, (C) α-amylase (BAN), (D) *Paenibacillus* sp F8 CGTase, (E) *P. stutzeri* maltotetraohydrolase. The detected peaks represent DP1 = glucose, DP2 = maltose, DP3 = maltotriose, DP4 = maltotetraose, DP5 = maltopentaose, DP6 = maltohexaose, DP7 = maltoheptaose, α-CD, β-CD, γ-CD = α-, β-, γ-cyclodextrin, respectively.

The HPAE-PAD is particularly well suited for the analysis of the time course of an enzymatic reaction, since formed products can be qualitatively detected. The formation of initial and end products by starch hydrolysis can be easily determined by comparison of chromatograms obtained at different time intervals. The elution conditions can be adjusted and results can be obtained within a short time. With the HPAE–PAD direct quantification of non-derivatised carbohydrates at low picomole values with minimal sample preparation can be performed. The detection is sensitive and consumes only small amounts of sample. Compared with the TLC technique, which is a cheap method requiring few materials and HPLC with RI-detection, HPAE–PAD is superior for the identification of the starch hydrolysis products formed by amylase activity. The three methods are listed according to reproducibility and separation of products according to their DP in Table 3.

*Conclusions.*—For activity determinations care should be taken in the choice of the saccharogenic assay and the use of saccharide reference. Comparison of the amylase activities published in the literature is difficult due to the use of different methods and saccharide references for determination of reducing sugar. A significant problem was shown using

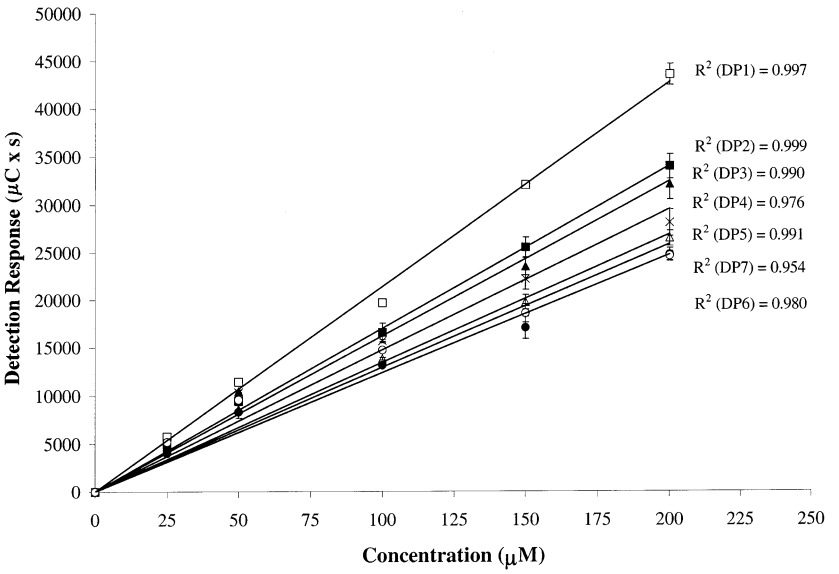


Fig. 4. Standard curves for HPAE–PAD for glucose and malto-oligosaccharides. □ Glucose, ■ Maltose, ▲ Maltotriose, X Maltotetraose, △ Maltopentaose, ● Maltohexaose, ○ Maltoheptaose.

the DNS and $CuSO_4$/biquinoline methods as they both gave different responses to glucose and maltose. For the DNS method [18] the actual product formed should be used as the reference, while other methods mainly need attention by the use of glucose as reference resulting in an overestimation of activity compared with malto-oligosaccharides. Only comparison between investigations using the same methods and even the same saccharide reference is recommended.

The identification of the starch hydrolysis products produced by the malto-oligosaccharide-forming amylases is an essential step in the determination of the activity and classification of the amylase. Chromatography methods, preferably HPAE–PAD can be used for the detection of these products. For detection of amylolytic activity on pure substrates of low DP the HPLC and the TLC techniques are appropriate. An improved separation of hydrolysis products can be obtained by HPAE–PAD within short analysis times. Initial products of high DP (up to DP 50) can also be detected by this method. Due to the high sensitivity of detection, this method is an excellent tool for following the starch hydrolysis over time. The present investigation showed that the response for similar molar concentrations of different malto-oligosaccharides decreases with increasing molecular size until DP6.

Malto - oligosaccharide - forming amylases have been named according to a predominant hydrolysis product formed at the beginning of the hydrolysis [32,34,35,43] or a hydrolysis product dominating at the steady state of the hydrolysis [44–46]. The steady state is obtained when the formed products are not changing by further hydrolysis [47] also described as end products. Hayashi et al. [34,35] described an amylase as a maltohexaose-forming amylase based on the initial product formation. An amylase of similar activity was described by Kim et al. [44] as a maltotetraose-forming amylase based on the end product formation. An indication of the stage of hydrolysis at which the specific product is produced could minimise the misleading first impression of very different enzymes.

Some malto-oligosaccharide-forming amylases have been found to form maltotriose [31], maltotetraose [48], maltopentaose [32], and maltohexaose [49] by an endo-type of action mode. However, the main part of the malto-oligosaccharide-forming amylases has been found to form starch hydrolysis products of a specific DP by an exo-type of action mode (Table 4). Exo-maltotetraohydrolase (E.C. 3.2.1.60) from *P. stutzeri* NRRL B 3389 [5] and exo-maltohexaohydrolase (E.C. 3.2.1.98) from *Klebsiella pneumonia* [7] have been classified and given systematic names as

suggested by studies of their hydrolytic action [6,53]. However, the nomenclature is not used systematically for malto-oligosaccharide-forming amylases throughout the literature. The maltohexaose-forming amylase from *Bacillus caldovelox* was found to cleave starch by an exo-type of action mode [52], still it was classified as an α-amylase which is not consistent with the exo-activity. Clearly, the action mode is of importance for classification of the malto-oligosaccharide-forming amylases.

We therefore suggest a nomenclature including the action mode of the enzyme, the predominant starch hydrolysis product formed and the stage of hydrolysis at which this product is formed. This way three different classes of amylases forming one predominant malto-oligosaccharide can be identified:

1. Exo - malto - oligosaccharide hydrolase should include amylases, which predominantly form a malto-oligosaccharide of a specific DP at the beginning and throughout the entire period of hydrolysis by an exo-action mode, as, e.g., the exo-maltotetraohydrolase found by Robyt and Ackerman [5].
2. Exo-malto-oligosaccharide-forming amylase for amylases which form a malto-oligosaccharide of specific DP as end product by an exo-action mode. This amylase would form an initial product which is further hydrolysed to the predominant end product determining the classification. This can be illustrated by the exo-maltotetraose forming amylase described by Kim et al. [44] initially forming maltohexaose which is further degraded to the end product maltotetraose.
3. Malto-oligosaccharide-forming α-amylase are amylases predominantly forming a malto-oligosaccharide of a specific DP by endo-action mode. This term should not include α-amylases forming a broad distribution in the DP of products as e.g. the *B. amyloliquefaciens* amylase.

Since glucose is not a malto-oligosaccharide glucose formers are excluded, while β-amylase still can be included as an exo-maltose hydrolase. Recently, this more precise terminology has been used [34,35,44,51,52], however with some inconsistency as indicated above.

## 3. Materials and methods

*Materials.*—Soluble starch, glucose, potassium–sodium tartrate, and $CuSO_4 \cdot 5H_2O$ were obtained from E. Merck KGa, Darmstadt, Germany. Maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose and maltoheptaose, 3,5-dinitrosalicylic acid (DNS), and 4,4′-dicarboxy-2,2′-biquinoline were obtained from Sigma Chemical Co., St. Louis, USA. α-, β- and γ-cyclodextrin were obtained from Wacher Chemie GmbH, Burghausen, Germany. Maltotetraose was prepared as de-

Table 4
Bacterial malto-oligosaccharide forming amylases of endo- and exo-action mode [a]

| Origin | Malto-oligosaccharide | Mode of action | Ref. |
|---|---|---|---|
| *Streptomyces griseus* NA-468 | maltotriose (DP3) | exo | [50] |
| *Bacillus subtilis* | maltotriose (DP3) | endo | [31] |
| *Bacillus* sp. MG-4 | maltotriose (DP3) | – | [53] |
| *P. stutzeri*NRRL B-3389 | maltotetraose (DP4) | exo | [5] |
| *Pseudomonas* sp.IMD353 | maltotetraose (DP4) | endo | [48,51] |
| *Bacillus* sp. GM8901 | maltotetraose (DP4) | exo | [44] |
| *Bacillus licheniformis* 584 | maltopentaose (DP5) | endo | [32] |
| Isolate 163-26 (DSMZ 5853) | maltopentaose (DP5) | exo | [42] |
| *Aerobacter aerogenes (K. pneumonia)* | maltohexaose (DP6) | exo | [7,8] |
| *Bacillus circulans* F-2 | maltohexaose (DP6) | exo | [33,43] |
| *B. circulans* G-6 | maltohexaose (DP6) | endo | [49] |
| *Bacillus* sp. H.167 | maltohexaose (DP6) | exo | [34,35] |
| *B. caldovelox* | maltohexaose (DP6) | exo | [52] |

[a] Examples are listed with origin, mode of action, formed products, and references.

scribed previously [54]. All other chemicals were unless stated otherwise of analytical grade. Amyloglucosidase (AMG, E.C. 3.2.1.3), a glucose producing amylase from *A. niger*, maltogenase (E.C. 3.2.1.-), a maltose producing amylase from *B. stearothermophilus* and a bacterial α-amylase (BAN, E.C. 3.2.1.1) from *B. amyloliquefaciens*, were all obtained from Novo Nordisk, Bagsværd, Denmark. Purified *Paenibacillus* sp. F8 CGTase was obtained as previously described [41]. Crude exo-maltotetraohydrolase (E.C. 3.2.1.60) was prepared as described by Robyt and Ackerman [5] and used without further purification.

*Determination of reducing sugars.*—For determination of reducing sugars standard curves of glucose and malto-oligosaccharides were prepared. All measurements were performed in triplicates. For the $CuSO_4$/biquinoline assay [26] standard curves of glucose, maltose, maltotriose, maltotetraose and maltoheptaose, respectively, in the concentration range 0.025–0.3 mM were prepared.

For the DNS method of Bernfeld [18] standard curves of glucose, maltose and maltotriose were prepared, respectively, in the concentration range 0.1–3.0, 5.0 and 10.0 mM. Determinations were done spectrophotometrically at 540 nm.

*Enzyme assay.*—2.0 mL soluble starch solution (1.25% (w/v)) in buffer (acetate buffer pH 5.0, phosphate buffer pH 7.0) and 0.5 mL enzyme solution (diluted to give a proper level of activity) was mixed to give a final concentration of 1% starch. The reaction was incubated at optimal temperature and stopped by boiling the mixture for 10 min. The formation of reducing sugars was determined in duplicates with the $CuSO_4$/biquinoline assay [26]. Maltose was used as a reference except for AMG where glucose was used.

*Determination of starch–iodine blue colour formation.*—An $I_2$–KI solution (0.20% (w/v) KI and 0.02% (w/v) $I_2$) was prepared according to Fuwa [23]. The starch–iodine blue colour formation was measured in duplicates with the following modifications. A sample of 500 μL was withdrawn from the enzymatic hydrolysis mixture of soluble starch at different time intervals. pH was adjusted by 250 μL 1.0 M HCl and 250 μL $I_2$–KI solution were added and mixed. Deionised water (4.0 mL) was added and mixing was repeated. The blue colour developed after 10 min at ambient temperature was measured spectrophotometrically at 600 nm.

*Analysis of hydrolysis products.*—Hydrolysis products of starch and reference malto-oligosaccharides (25–200 μM) were detected using high performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD). A CarboPac PA-1 column (Dionex Corporation, Sunnyvale, CA, USA) was used with a gradient of 1.0 M NaOAc from 0 to 60% over 30 min in 100 mM NaOH at a flow rate of 1.0 mL/min on a Dionex DX-300 system (Dionex Corporation, Sunnyvale, CA, USA). Starch hydrolysis products were identified by comparison of their retention times with glucose, maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose, maltoheptaose, α-, β- and γ-CDs. Since the retention times of homologous linear malto-oligosaccharides increases with the DP, linear malto-oligosaccharides of intermediate DP could easily be identified [40].

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